Rapid Assay of Choline in Foods Using Microwave Hydrolysis and a Choline Biosensor

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A fast procedure for the determination of choline in food was developed by coupling a microwave hydrolysis procedure with an O_2/choline oxidase-based electrochemical biosensor. Time and temperature were varied to select the best conditions for the microwave hydrolysis. Results have been compared with those found by the traditional method, constituted by hydrolysis at 70 °C followed by enzymatic−colorimetric assay. Data obtained by the biosensor method correlated well with the enzymatic−colorimetric assay ($R^2 = 0.998$). Microwave versus traditional hydrolysis gave a good correlation both with the colorimetric and with biosensor procedures with a relative error below 6%. The method is sensitive and selective enough to be used for a wide variety of food items reducing remarkably the analysis time.

Keywords: *Choline; microwave hydrolysis; biosensor; amperometry; food*

INTRODUCTION

Choline is a dietary requirement of several animal species (dog, cat, rat, and guinea pig), and only recently (Fomon and McCormik, 1993) it was suggested that a dietary source of choline may be required for adult humans.

Choline is an important component of phospholipids (lecithin and sphingomyelin), it is required for the synthesis of the neurotransmitter acetylcholine, it acts as a source of labile methyl groups, and it is a component of pulmonary surfactant (Zaloga and Bortenschlager, 1994).

This substance is normally produced in human tissues in sufficient amounts to meet human needs, and it is classified as being "vitamin-like". The endogenous synthesis of choline, however, requires adequate amounts of the amino acids serine and methionine, along with adequate amounts of folic acid and vitamins B_{12} and B_6 (Zaloga and Bortenschlager, 1994). Choline is widely distributed in foods; the requirement is generally satisfied by both dietary and endogenous sources, although choline deficiency has been reported. The most common signs of choline deficiency are fatty liver and hemorrhagic kidney necrosis (Chan, 1984). Evidence for free radical activity in liver with choline deficiency is reported, and this may be related to the carcinogenesis process (Farber and Ghoshal, 1998).

Neonates need a dietary supply of choline, and, for this reason, the American Academy of Pedriatics (AAP, 1985) has recommended that infant formulas contain at least 7 mg of choline/100 kcal. Choline is ingested mainly in the form of phosphatidylcholine rather than free base. Choline chloride and choline bitartrate are added to infant formulas and milk products to ensure the presence of choline at levels found in milk (Chan, 1984).

Recently, an Adequate Intake (AI) of choline has been established by the Institute of Medicine, Food and Nutrition Board (1998). The AI is a recommended daily intake level based on observed or experimental determined approximations of nutrient intake by a group of healthy people. The AI is used when recommended dietary allowances (National Research Council, 1989) cannot be determined because sufficient data are lacking. It is worth noting that the AI for choline in infants ranges from 125 to 150 mg/day, whereas it is 550 mg/day in adult males and 425 mg/day in adult females. For these reasons, a fast and versatile methodology to determine choline contents in different food items is needed. The estimation of choline is difficult because of the various forms in which it may occur, and numerous techniques have been applied to choline determination. The "classical" method involves the precipitation of choline as a reinecke salt in a colorimetric reaction (Casson and Griffin, 1959; Martinez, 1983). Other methods include microbiological assay (Lied and Braekkan, 1975), gas chromatography (Saucerman et al., 1984), and HPLC (Ikarashi and Maruyama, 1993). To determine choline, enzyme-based methods have been used coupled with colorimetric (Takayama et al., 1977), fluorescence (Fleming and Gadsden, 1987), UV (Boehringer, 1984), or amperometric (Campanella, 1986; Karube et al., 1979; Masoom et al., 1990) detection. Recently an enzymatic−colorimetric assay (Woollard and Indyk, 1990) has been used in a collaborative study for the AOAC Official Methods Program for choline determination in infant formulas and milk.

Various procedures for sample preparation have been applied to determine choline in different food items such as solvent extraction (Boix Montanes and Permanyer

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Fabregas, 1997), and enzyme hydrolysis (Kotsira and Clonis, 1998), hydrolysis in methanolic KOH (Zhang and Wilson, 1998) or in HCl (Woollard and Indyk, 1990).

Microwave hydrolysis has been already successfully used by our group to rapidly hydrolyze food samples for the determination of total amino acids (Marconi et al., 1995), lysine (Marconi et al., 1996), furosine (Acquistucci et al., 1996), and vitamin B_2 (Panfili et al., 1994). In this work a microwave procedure for choline determination has been optimized and coupled with a biosensor. Both the hydrolysis procedure and the choline detection were optimized and applied to measure choline in commercial food samples; validation was obtained by comparison with the enzymatic−colorimetric assay (Woollard and Indyk, 1990) used in the collaborative study for the *AOAC Official Methods* (AOAC, 2000).

MATERIALS AND METHODS

Chemicals. Choline bitartrate, phospholipase D (type II), peroxidase (type I), BSA, glutaraldehyde, and choline oxidase were from Sigma Chemical Co. (St. Louis, MO). Deionized water was purified with a Milli-Q water purification system (Millipore, Bedford, MA). Gas-permeable membranes were from AMEL (Milan, Italy), polycarbonate membranes (0.03 μm porosity) were purchased from Nuclepore (Pleasanton, CA), and nylon net was from a local shop. All other reagents were of analytical grade and were purchased from Carlo Erba (Milan, Italy).

Samples. All samples were purchased in local supermarkets. Pasta samples were ground in a Buhler-Miag model ML1204 grinder (Uzwil, Switzerland); yolk sample was obtained by pooling a standard package of eggs ($n = 6$). NIST is a Standard Reference Material (Infant Formula 1846) coming from the National Institute of Standards and Technology (Gaithersburg, MD).

Equipment. The microwave oven used was a microwave hydrolysis system CEM Corp. (Matthews, NC), model 2000, maximum power, 630 ± 50 W, and magnetron frequency, 2435 MHz. The system was equipped with probes to detect and control the pressure and temperature inside the sealed vessel so as to regulate hydrolysis conditions via magnetron power output control. PFA Teflon vessels (l00 mL volume) were used. Spectrophotometric measurements were performed using a Beckman DU-7 (Palo Alto, CA). A Universal Sensor (Metairie, LA) Pt electrode polarized at −700 mV versus Ag/AgCl was used for the amperometric measurements; this was connected to a Universal Sensors amperometric biosensor detector (ABD). The signal was also monitored with an AMEL model 868 chart recorder. The biosensor assembly comprised a choline probe realized by placing the gas-permeable membrane, the choline oxidase membrane, and the polycarbonate membrane on the electrode jacket provided by the manufacturer. The jacket was then filled with 0.1 M KCl (supporting electrolyte) and mounted onto the Pt probe with the gas-permeable membrane in contact with the working electrode surface. The enzymatic membrane was prepared using a BSA−glutaraldehyde covalent immobilization and nylon net as physical support. Fifteen microliters of 0.1 M phosphate buffer, pH 7.0, containing 1 mg of choline oxidase (10 units), 0.5 mg of BSA, and 0.5% (w/v) glutaraldehyde was placed onto 1 cm^2 of nylon net and allowed to react for 1 h at room temperature. The resulting membrane was washed in the working buffer (0.1 M glycine, pH 8.0) and used to assemble the biosensor.

Sample Preparation. The sample, estimated to contain between 1.0 and 10.0 mg of choline, was weighed (ranged from 0.2 to 20.0 g). Thirty milliliters of 1 M HCl for solid samples or 10 mL of 3 M HCl for liquid samples was added. A traditional hydrolysis for 3 h at 70 °C was carried out according to the method of Woollard and Indyk (1990). For the hydrolysis optimization of the microwave equipment, which allows detection and control of the different operational conditions (magnetron power, pressure, time, and temperature), is recommended. In this test, six lined PFA Teflon digestion vessels were used. The samples were put into the vessels and left to hydrolyze under nitrogen with 30 mL of 1 M HCl. The samples were submitted to four different (A−D) heating cycles. The four different microwave hydrolysis programs are different in temperature/time parameters:

	program			
	A	B	C	D
power (% 630 W)	80	80	80	80
time (min)	30	10	5	5
temp (°C)	70	100	110	120
pressure maximum (psi)	40	40	40	40

After the hydrolysis steps, the vessels were cooled in an ice bath, and then the pH was adjusted to 3.8−4.0 with 50% w/w sodium hydroxide solution. The hydrolysate was transferred to 50 mL volumetric flasks and brought to volume with water. An aliquot of hydrolysate was filtered and stored in the dark at 4 °C for up to 3 days prior to analysis.

Enzymatic—Colorimetric Determination. Enzymatic−colorimetric method was performed according to the method of Woollard and Indyk (1990). In the hydrolyzed samples, free choline was subjected to choline oxidase, liberating hydrogen peroxide. In the presence of peroxidase, phenol is oxidized and a colored compound formed with 4-aminoantipyrine.

Absorbance is measured at 505 nm and choline content calculated by interpolation from a standard calibration graph.

Biosensor Determination. The choline probe was left to equilibrate in 3 mL of working buffer (0.1 M glycine, pH 8.0) placed in a thermostated glass cell at 25 °C until a stable baseline was achieved. Sample solutions, diluted as appropriate in the working buffer, were then added, and the decrease in current was recorded until a steady state was attained. A standard solution of choline was injected in the cell and used as single standard addition to calculate the concentration of choline in solution. The probe was stored at 4 °C in a 1% DEAE-Dextran−5% lactitol solution when not in use.

RESULTS AND DISCUSSION

In this paper, a modified assay based on the original method from Woollard and Indyk (1990) to quantify total choline in foods is presented. This method is based on acid hydrolysis of the food samples carried out at 70 °C for 3 h and enzymatic spectrophotometric detection of choline using choline oxidase and peroxidase. To decrease the analysis time and the cost per analysis, we used an O_2-based choline biosensor and microwave hydrolysis.

Choline Biosensor. Amperometric measurement of choline has been already reported by different authors by immobilization of the enzyme choline oxidase onto an electrode surface able to detect the consumption of O_2 or the production of H_2O_2 during the enzymatic reaction. Most of the work has been devoted to environmental samples for the measurement of pesticides and herbicides through inhibition of the acetylcholinesterase activity (Troianowicz and Hitchman, 1996). The application on food samples requires the coimmobilization of phospholipase to liberate the conjugated choline in the sample. This leads to an increase in the response time and/or a drastic decrease in sensitivity of the assay (Karube et al., 1979; Masoom et al., 1990).

Our goal in this work was to use the excellent analytical behavior of the monoenzymatic probe in conjunction with a rapid and efficient microwave hydrolysis step. The amperometric probe selected was O_2-based because of the selectivity and simplicity of the Clark type oxygen electrode. Although measurement with an H_2O_2-based probe is intrinsically more sensitive

Table 1. Choline Contents (Milligrams per 100 g) in the Studied Products[a]

sample	enzymatic−colorimetric (A)	RSD $n = 3$	biosensor (B)	RSD $n = 3$	$[(A - B)/A]$ %
pasteurized milk	12.6	1.4	13.8	0.7	9.6
soy lecithin in granules	2333.6	1.7	2270.0	1.2	2.7
soy-based infant formula	39.3	3.8	36.5	3.9	7.2
milk powder	84.7	3.3	93.0	4.5	9.7
pasta	153.8	0.6	149.2	0.5	3.0
pasta with eggs	230.4	5.1	210.0	3.4	8.9
dietetic meal	154.5	1.0	166.0	7.7	7.4
NIST	132.2	1.2	141.6	4.9	7.1
yolk	1045.4	1.3	945.5	2.2	9.6
egg powder	1305.3	8.6	1282.2	10.4	1.7
enriched milk for children	9.6	8.1	10.2	6.3	6.3

[a] Comparison between biosensor and enzymatic−colorimetric determinations.

Table 2. Comparative Study between Choline Values (Milligrams per 100 g) Obtained by Traditional Method (A) and Combinations of Microwave and Biosensor (C) or Microwave and Colorimetric (D)

sample	70 °C × 3 h colorimetric (A)	RSD $n = 3$	MW 110 °C × 5 min biosensor (C)	RSD $n = 3$	MW 110 °C × 5 min colorimetric (D)	RSD $n = 3$	$[(C - A)/A]$ %	$[(D - A)/A]$ %
egg powder	1305.3	8.6	1227.2	7.2	1230.0	5.1	6.0	5.8
enriched milk for children	9.6	8.1	9.9	8.6	9.0	0.8	3.2	6.3
pasta with eggs	230.4	5.1	243.5	12.5	237.5	1.3	5.7	3.1

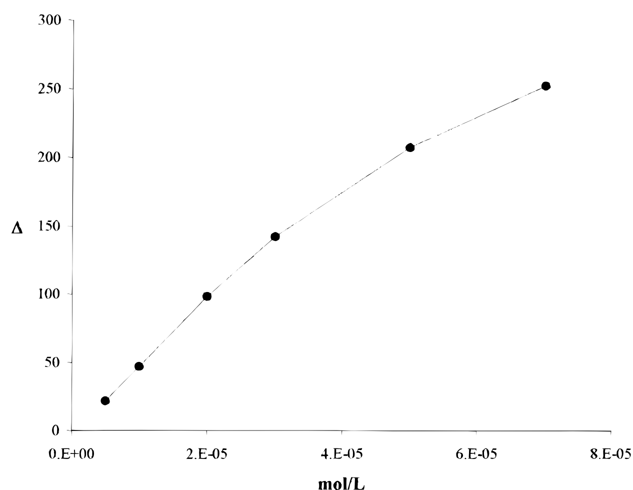

Figure 1. Calibration curve of choline using the choline biosensor.

and has a larger dynamic range, particular attention must be paid to obtain a selective response for H_2O_2 (by the use of laboratory-made membranes or interference rejecting polymers onto the electrode surface).

A typical calibration curve for choline obtained using the biosensor assembled as described under Materials and Methods is reported in Figure 1. The response to choline was found to be linear in the 5−50 μmol/L range ($R^2 = 0.994$) with an RSD of 4−5%. Response time (95% of the steady state) was 3−4 min.

Table 1 reports the choline content in food samples measured with colorimetric−enzymatic (A) and biosensor methods (B) after 3 h of hydrolysis at 70 °C. Samples exhibited a wide range in choline content (9.6−2333.6 mg/l00 g) due to the different compositions of the analyzed foods. The data are generally in agreement with those previously published about choline contents (Woollard and Indyk, 1990; Campanella et al., 1986; Chan, 1984; Indyk et al., 1993).

In particular, data on pasteurized milk (12.6 and 13.8 mg/100 g) and milk powder (84.7 and 93.0 mg/100 g) are similar to those reported by other authors, whereas data about soy lecithin in granules (2333.6 and 2270.0 mg/100 g) and soy-based infant formula (39.3 and 36.5 mg/100 g) are comparable with the data declared in the labels.

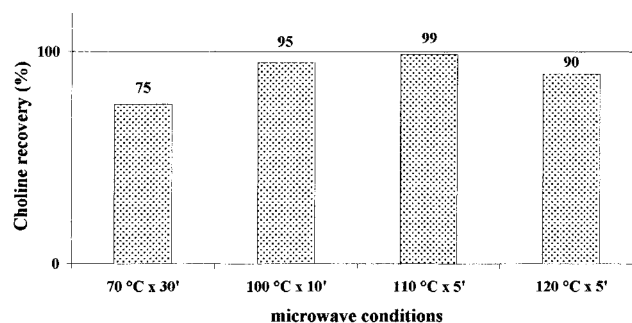

Figure 2. Recovery (percent) of choline contents in pasta sample, using different microwave hydrolysis programs, compared to the traditional method (100%).

The analytical performances of the two methods were similar as demonstrated by the mean values, by the relative standard deviation (RSD), and by the relative error (RE%) reported in Table 1. The regression equation obtained by plotting the spectrophotometric versus the amperometric method was $y = 0.969x + 1.923$ with $R^2 = 0.999$.

Microwave Hydrolysis. As already mentioned in the Introduction the microwave-based hydrolysis procedure has been successfully used with different analytes. Hydrolysis of choline was studied initially on a pasta sample (2.5 g) containing ∼3−4 mg of choline.

This sample was submitted to four different (A−D) heating cycles described under Materials and Methods. For this procedure microwave equipment that allows detection and control of the different operational conditions (magnetron power, pressure, time, and temperature) is recommended. Data are reported in Figure 2 as recovery compared to the value obtained in the same samples by using the method of Woollard et al. (1990).

The use of a hydrolysis procedure at a temperature of 70 °C for 30 min (program A) seems not to be adequate to fully hydrolyze the sample. In fact, an incomplete hydrolysis due to insufficient temperature was evident (75% recovery).

Better recoveries were obtained at a temperature of 100 °C. When 110 °C for 5 min (program C) is applied, a total recovery (99%) of choline is obtained. The

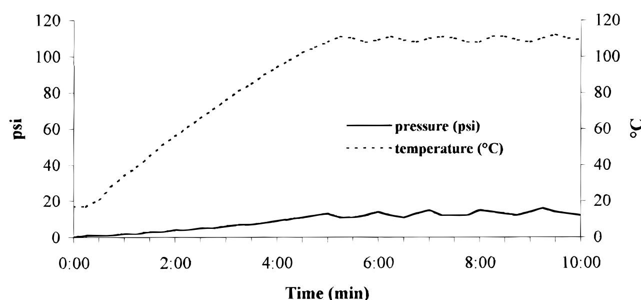

Figure 3. Typical pressure/temperature diagram of the pasta sample by the selected irradiation program (110 °C for 5 min).

irradiation program D (120 °C for 5 min) proved to be too severe for choline analysis. A typical pressure/temperature diagram of the pasta sample is shown in Figure 3 for the conditions of program C, which was selected for further measurements.

In Table 2 data obtained with hydrolysis at 70 °C for 3 h and the selected microwave hydrolysis (110 °C for 5 min), measured with both spectrophotometric (*A*) and amperometric (*B*) detection, are reported. Three different sources of food were used (egg powder, pasta, and milk) with different choline contents.

Data correlated well with an RE% not higher than 6.3%, proving that the use of the microwave plus biosensor procedure is suitable for choline determination in all of the studied samples.

The use of microwave plus biosensor is versatile and can be applied to different food items also in the presence of low levels of choline.

It has to be stressed that the sample dilution used in the biosensor procedure was in the 1:10 to 1:500 range depending on the choline content of the sample. Such significant dilution avoids further neutralization steps after the acid hydrolysis, thus reducing the total analysis time of choline to 15–30 min. This procedure seems to be suitable for the rapid measurement of choline, maintaining the same sensitivity and accuracy of the enzymatic spectrophotometric method with the typical advantages of the use of an amperometric biosensor such as reusability of the enzyme, no use of additional reagents, and possibility to measure turbid samples with no pretreatment.

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